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Newcastle disease outbreaks in Kazakhstan and Kyrgyzstan during 1998, 2000, 2001, 2003, 2004, and 2005 were caused by viruses of the genotypes VIIb and VIId

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Abstract Newcastle disease virus (NDV) infects domesticated and wild birds throughout the world, and infections with virulent NDV strains continue to cause disease outbreaks in poultry and wild birds. To assess the evolutionary characteristics of 28 NDV strains isolated from chickens in Kazakhstan and Kyrgyzstan during 1998, 2000, 2001, 2003, 2004, and 2005, we investigated the phylogenetic relationships among these viruses and viruses described previously. For genotyping, fusion (F) gene phylogenetic analysis (nucleotide number 47–421) was performed using sequences of Kazakhstanian and Kyrgyzstanian isolates as compared to sequences of selected NDV strains from GenBank. Phylogenetic analysis demonstrated that the 14 newly characterized strains from years 1998 to 2001 belong to the NDV genotype VIIb, whereas the 14 strains isolated during 2003–2005 were of genotype VIId. All strains possessed a virulent fusion protein cleavage site (R-R-Q-R/K-R-F) and had intracerebral pathogenicity indexes in day-old chickens that ranged from 1.05 to 1.87, both properties typical of NDV strains classified in the mesogenic or velogenic pathotype.

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Introduction

Newcastle disease (ND) is regarded throughout the world as one of the two most important diseases of poultry and other birds, the other disease being highly pathogenic avian influenza [1]. ND as defined by the World Organization for Animal Health (OIE) is caused by an infection of birds with virulent strains of avian paramyxovirus serotype 1 (APMV-1), a term synonymous with ND virus (NDV) [1]. The importance of ND is not only due to the devastation ND virus (NDV) infections may have on infected birds due to the potential of causing flock mortality rates of up to 100% but also the economic impact that may ensue due to trading restrictions and embargoes placed on regions and countries where outbreaks have occurred [2, 3]. Infections with NDV strains of low virulence are also widespread in birds and include those strains used widely as live vaccines in poultry. Kaleta and Baldauf [4] concluded that NDV infections have been established in at least 241 species of birds representing 27 of the 50 orders of the Class Aves. The characterization of the virulence of new isolates is important for differentiation of virulent from low-virulence strains. In contrast to infection of birds with a virulent NDV strain, an infection with a low-virulence NDV is not defined as ND, therefore that occurrence does not require reporting to national or international regulatory agencies [1].

Four main global streams of infection have been recognized in the history of ND panzootics in chickens and other bird species [1, 5]. The first recorded panzootic

started in the Southeast Asia in the mid-1920s and it took about 30 years to spread throughout the world. At least three genotypes (II, III, and IV) were involved in the first panzootic [6]. A second panzootic is recognized to have started in the Middle East in the 1960s and to have spread to most countries by the early 1970s. This panzootic prompted the development of improved vaccines and vaccination protocols that combined with implementation of sanitary measures brought the disease under control in North America and in some European countries. In the larger part of the world, however, especially where rural chicken breeding is dominant, ND had become endemic. Monoclonal antibody analysis established a close relationship of NDV strains of the second panzootic with isolates from imported psittacines [7]. This was later confirmed by genetic analysis of the fusion (F) protein gene and the classification of these isolates as genotype V [8, 9].

The start and spread of the third panzootic are unclear. Use of improved vaccines had resulted in improved disease control, but vaccination did not prevent infection and the transmission of virus from infected vaccinates. Subsequent characterization of those NDV isolates recovered after the second panzootic revealed further genetic variation. The presence of genotype VII was identified in Taiwan and Indonesia in the 1980s [6, 9]. The presence of isolates of genotype VIIb in southern Africa and their involvement in the NDV outbreaks of the 1990s was first described by Herczeg et al. [10]. The occurrence of other isolates characterized as subtypes of genotype VII have also been reported: VIIa in the Far East and Europe [11, 12]; VIIb in the Far East, Middle East, Europe [13–16], India, and southern Africa [17, 18]; VIIc in the Far East and Europe; and VIId in the Far East and South Africa [6, 18].

The fourth panzootic is attributed to a NDV variant that infects primarily pigeons and doves [3] and is now characterized as genotype VI [8, 9]. The variant virus was first identified in isolates recovered in the Middle East in the late 1970s, spread to Europe in the 1980s, and was responsible for outbreaks in unvaccinated chickens infected from feed contaminated by infected pigeons in Great Britain during the mid-1980s. Although the timeframe overlaps the occurrence of the third panzootic, the variant virus remains enzootic in pigeons and is a continuing threat to establish infections in poultry.

Because of the widespread use of ND vaccines in Kazakhstan and Kyrgyzstan, an epizootic of ND has not occurred in recent years; however, sporadic outbreaks of the disease still exist. In a previous study [16], 10 Kazakhstanian viruses isolated during 1998 were analyzed phylogenetically and found to be genotype VIIb. In this study, 28 additional NDV isolates obtained from outbreaks occurring between 1998 and 2005 in Kazakhstan and Kyrgyzstan are characterized molecularly, and the epidemiology of ND is evaluated on the basis of molecular analysis of the F protein gene of those isolates.

Materials and methods

Virus isolation

Twenty-eight NDV isolates were collected between 1998 and 2005 in the Almaty (including Konyr and Taldykorgan) and Astana regions of Kazakhstan and the Bishkek region of Kyrgyzstan. All strains were isolated from different chicken flocks during ND outbreaks. NDV isolates were recovered from cloacal and/or tracheal swabs and postmortem material taken from ill or dead birds. Initial isolation of the virus was performed in 9–10-days-old embryonated chicken eggs (ECE). The isolates were identified in standard hemagglutination inhibition and neuraminidase inhibition tests using specific antisera to the reference strains of NDV (avian paramyxovirus type 1, APMV-1), other APMV of types 2–9, and 15 subtypes of influenza A virus. The strains were labeled with the codes of country and a serial number in order of receipt (Table 1). Allantoic fluids were harvested from ECE inoculated with the viruses and used as a stock for sequence analysis and other virus characterization tests [16].

Mean death time test

Virus-infective allantoic fluid was diluted 10-fold in PBS, pH 7.2 from 10^{-6} to 10^{-10} for ECE inoculation. Chicken embryo mean death time (MDT) induced by a minimal lethal virus dose was determined by standard procedures [19], and isolates with MDT up to 60 h were referred as velogenic, from 61 to 90 h as mesogenic, and more than 90 h as lentogenic.

ICPI test

The intracerebral pathogenicity index (ICPI) of NDV isolates was determined by intracerebral inoculation of 1-day-old chickens with 50 µl of NDV-containing infectious allantoic fluid with a HA titer $>2^4$ (>1/16) diluted 1:10 in PBS without antibiotics. The birds in each inoculum group were examined every 24 h for 8 days and scored as to the number of normal, sick, and dead. ICPI was calculated from the daily scores according to OIE methods [20]. A valid test was based on the absence of disease in inoculated controls. NDV strains with an ICPI below 0.7 were referred as lentogenic, above 1.5 as velogenic, and strains with intermediate ICPI value have been classified as mesogenic [19, 20].

Table 1 Biological and molecular characteristics of virulent NDV isolates recovered from chickens in Kazakhstan and Kyrgyzstan during 1998, 2000, 2001, 2003, 2004, and 2005

Strain abbreviation	ICPI	MDT	Fusion protein cleavage site (molecular pathotyping)	Accession number
KAZ-24-98	1.76	56.0	^{112} RRQRR^{116}* F^{117}	FJ385767
KAZ-42-98	1.5	52.0	^{112} RRQRR^{116}* F^{117}	FJ385770
KAZ-32-98	1.76	53.6	^{112} RRQRR^{116}* F^{117}	FJ385768
KAZ-37-98	1.06	73.6	^{112} RRQRR^{116}* F^{117}	FJ385769
KAZ-53-98	1.56	58.8	^{112} RRQRR^{116}* F^{117}	FJ385771
KAZ-55-00	1.85	52.8	^{112} RRQRR^{116}* F^{117}	FJ434381
KAZ-61-00	1.60	59.2	^{112} RRQRR^{116}* F^{117}	FJ434382
KAZ-64-00	1.07	66.4	^{112} RRQRR^{116}* F^{117}	FJ434383
KAZ-68-00	1.74	53.6	^{112} RRQRR^{116}* F^{117}	FJ434384
KAZ-101-01	1.08	67.2	^{112} RRQRR^{116}* F^{117}	FJ434385
KAZ-125-01	1.75	48.0	^{112} RRQRR^{116}* F^{117}	FJ434386
KAZ-126-01	1.41	65.8	^{112} RRQRR^{116}* F^{117}	FJ434387
KAZ-127-01	1.85	57.1	^{112} RRQRR^{116}* F^{117}	FJ434388
KAZ-128-01	1.87	48.0	^{112} RRQRR^{116}* F^{117}	FJ434389
KAZ-341-03	1.5	60.0	^{112} RRQKR^{116}* F^{117}	FJ434390
KAZ-342-03	1.6	53.6	^{112} RRQKR^{116}* F^{117}	FJ434391
KAZ-343-03	1.72	44.6	^{112} RRQKR^{116}* F^{117}	FJ434392
KAZ-344-03	1.75	56.6	^{112} RRQKR^{116}* F^{117}	FJ434393
KAZ-346-03	1.87	46.4	^{112} RRQKR^{116}* F^{117}	FJ434394
KAZ-540-04	1.8	59.8	^{112} RRQKR^{116}* F^{117}	FJ434395
KAZ-541-04	1.61	49.6	^{112} RRQKR^{116}* F^{117}	FJ434396
KAZ-543-04	1.72	53.6	^{112} RRQKR^{116}* F^{117}	FJ434397
KAZ-545-04	1.67	56.8	^{112} RRQKR^{116}* F^{117}	FJ434398
KYR-1014-05	1.51	45.6	^{112} RRQKR^{116}* F^{117}	FJ434399
KYR-1015-05	1.35	68.8	^{112} RRQKR^{116}* F^{117}	FJ434400
KYR-1019-05	1.5	52.8	^{112} RRQKR^{116}* F^{117}	FJ455441
KYR-1021-05	1.5	41.6	^{112} RRQKR^{116}* F^{117}	FJ455442
KYR-1024-05	1.5	40.8	^{112} RRQKR^{116}* F^{117}	FJ455443

Sequencing and phylogenetic analysis

RNA was extracted from infective allantoic fluid with an RNeasy Mini Kit (QIAGENE, GmbH, Germany) utilizing the manufacturer's recommended procedures to obtain total RNA preparations.

Reverse transcription was completed using Moloney murine leukemia virus (MMLV) (Promega, USA) in 5 μl of reaction mixture (2.7 μl sample, 0.735 μl water, 1 μl 5× buffer for reverse transcriptase (Promega, USA), 0.19 μl 2 mM mixture of dNTPs, 0.25 μl 20 OE primer 614F, and 0.125 μl MMLV) at 37°C for 60 min. Polymerase chain reaction (PCR) was completed in a 25 μl reaction mixture (1 μl DNA matrix, 1 μl *Taq* polymerase, 1 μl 20 OE direct and reverse primers, 2.5 μl 2 mM mixture of dNTPs, 2.5 μl 10× buffer for PCR (Promega, USA), 16 μl water) for 30 cycles in a thermocycler (Eppendorf, USA) at the following temperatures/times: 94°C/25 s, 55°C/25 s, and 72°C/90 s. PCR was completed with primers 614F and 1356R. In case of a low concentration of RNA/cDNA detection and absence of a PCR fragment, a second round of PCR with primers 1005F and 601R was completed (temperatures/times: 94°C/25 s, 53°C/25 s, and 72°C/60 s for 35 cycles). If detection was positive, two sets of second round PCR were carried out with primers 614F/1082R and 1005F/1356R (temperatures/times: 94°C/25 s, 53°C/25 s, and 72°C/90 s for 35 cycles). Primers 1045F, 614F, 601R, 1082R, 480F, 1356R, and 1005 F used in this research were described earlier [16]. Primer 614F was used for transcription, and the other primers were used for sequencing.

The sequence of PCR products was obtained by using an ABI PRISM BigDye™ Terminator cycle sequencing reaction kit (Applied Biosystem) according to the manufacturer's instruction with an ABI 310™ automated

sequencer. PCR products were sequenced in both directions. The sequenced fragments of the F gene were compiled and edited using a Lasergene sequence analysis software package (DNA Star software version 5.0, Madison, WI, USA). Phylogenic analysis was conducted with MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0 to complete comparisons of the nucleotide sequences of our isolates with those of ND viruses obtained from the GenBank database [1, 6, 9–18, 21–34] The nucleotide regions used in the phylogenic analysis were fragments of F gene (from 47 to 374). The nucleotide sequences were deposited in GenBank and filed under the accession numbers: FJ385767–FJ385771, FJ434381–FJ434400, and FJ455441–FJ455443 (Table 1).

Results

Virus isolation

Twenty-eight NDV isolates were recovered and all were virulent by OIE standard criteria, a fusion protein cleavage site with a virulent sequence and an ICPI of equal or greater than 0.7) (Table 1). Of the 28, 24 were velogenic, MDT $\leq$ 60 h and ICPI $\geq$ 1.5 and 4 were mesogenic, MDT $>$ 60 h and ICPI $<$ 1.5.

Nucleotide sequence and similarity analysis

The nucleotide sequences of an F gene fragment of the 28 new isolates (Table 1) were compared with the 10 Kazakhstan isolates published earlier [16]. Amplification and analysis of a 374-bp fragment from ATG with primers 614F and 1082R revealed no deletions or insertions in any strains, but there were several point mutations that produced only a small number of nonsynonymous amino acid changes (Fig. 1). Sequence similarities were associated with year of isolation and separated the isolates into three groups, 15 isolates from 1998, 9 isolates from 2000 and 2001, and 14 isolates from 2003 through 2005. The typical strains in the first subgroup were KAZ-22-98 and KAZ-38-98; in the second subgroup—KAZ-55-00 and KAZ-101-01 and in the third subgroup—KAZ-342-03 and KAZ-541-04.

Alignment of the predicted amino acid sequences

The alignment of the predicted amino acid sequence of that 374-bp fragment of the 28 isolates from Kazakhstan and Kyrgyzstan (Table 1) and reference strains of eight genotypes is presented (Fig. 1). All have at least a pair of basic amino acids (arginine, R or lysine, K) at residues 115 and 116, a phenylalanine (F) at residue 117, and a basic amino acid (R) at 113 typical of the viruses that demonstrate high virulence for chickens [2]. In this study, the fusion cleavage site sequence of the 14 isolates recovered through 2001 all had the same residues ^{112} RRQRR^{116}* F^{117} in contrast to the 14 isolates recovered during 2003–2005 which all contained the residues ^{112} RRQKR^{116}* F^{117}. In addition to differences at the fusion cleavage site, there was further differentiation of the isolates into either genotypes VIIb or VIId by having either F^{19} or V^{19} (valine) typical of genotype VIIb or A^{11} (alanine), L^{28} (leucine), V^{52}, and K^{101} typical of genotype VIId viruses (Fig. 1). Isolates KAZ-42-98 and KAZ-53-98 differed from the other 1998 Kazakhstan isolates by having a serine (S) rather than proline (P) at position 10. Two other differences noted in previous 1998 isolates from Kazakhstan [16] included isoleucine (I) rather than P at position 4 of KAZ-39-98 and P rather than L at position 13 of KAZ-34-98. The sequence of all of the 2003–2005 isolates included V^{121}.

Phylogenetic analysis

A phylogenetic tree based on a 374-bp region of the F gene of 54 NDV isolates representing the IX genotypes of NDV including subgroups a, b, c, and d of genotype VII (Fig. 2) is similar to the results of previous phylogenetic analysis [9–11]. The 54 strains included in the analysis comprised six isolates from Kazakhstan that represent each of the similarity groups identified in the new isolates and the previously characterized strains [16] and 48 reference strains of all IX NDV genotypes. Although the 1998 Kazakhstan isolates (KAZ-22-98 and KAZ-38-98) differ from the Kazakhstan 2000 and 2001 isolates (KAZ-55-00 and KAZ-101-01) at fusion protein amino acid residue 19, all 1998, 2000, and 2001 isolates are most like FIN-1-96 and group with genotype VIIb isolates. In contrast, all of the 2003–2005 isolates from both Kazakhstan and Kyrgyzstan, represented by KAZ-342-03 and KAZ-541-04, are highly similar to the genotype VIId viruses China-BIF4 and China-GIF3.

Discussion

The first ND outbreaks in Kazakhstan and Kyrgyzstan were recorded in 1980 [35]. Although large-scale ND outbreaks have been controlled by the widespread use of ND vaccines, ND apparently either became endemic in many chicken flocks or the virus has been reintroduced from other infected birds. The consequence was the occurrence of outbreaks of clinical ND seen as a respiratory, neurological, and/or enteric disease typical of those cases that yielded the isolates characterized in this study [36].

All the isolates in the present study (Table 1) were found to be virulent based on the standard criteria MDT,

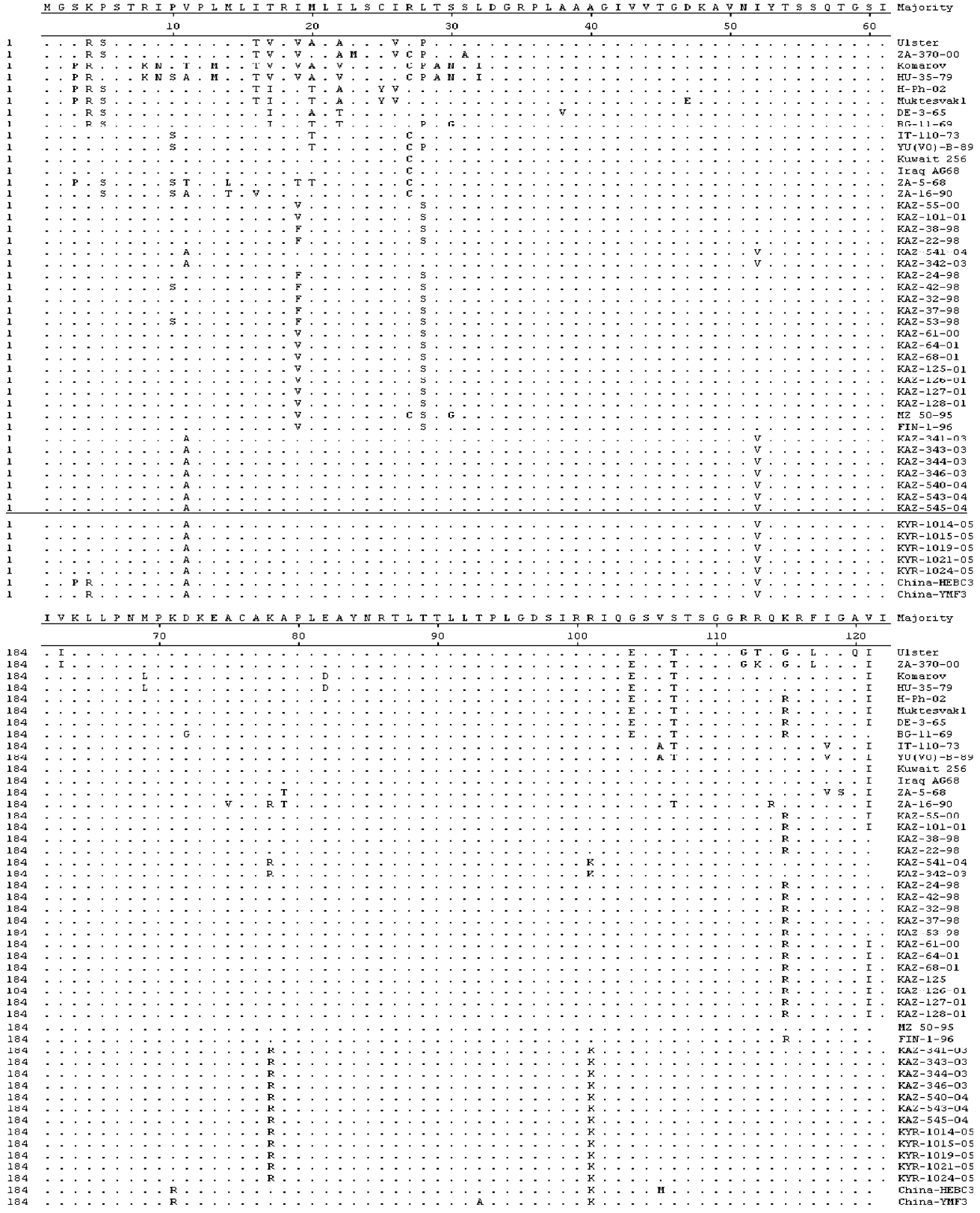

Fig. 1 Alignment of the predicted amino acid sequences of F proteins of 28 NDV isolates from Kazakhstan and Kyrgyzstan and 18 reference strains (eight genotypes)

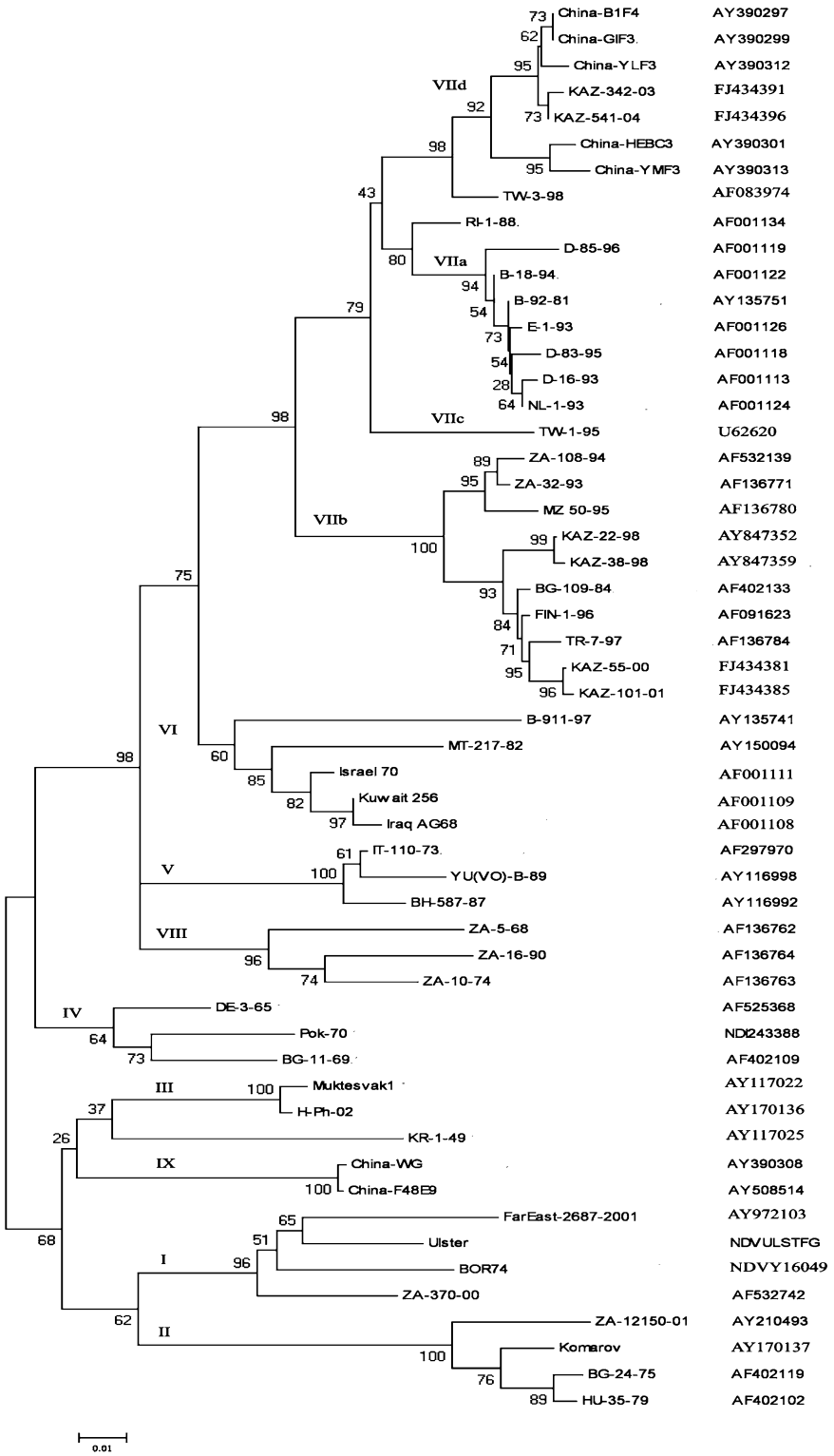

Fig. 2 Evolutionary relationships of 54 NDV strains based on a 374-bp (position 47–421 nt) region of the F gene. The evolutionary history was inferred using the Neighbor-Joining method [31]. The bootstrap consensus tree inferred from 1000 replicates [32] is taken to represent the evolutionary history of the taxa analyzed [32]. The evolutionary distances were computed using the Maximum Composite Likelihood method [33] and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 [34]

ICPI, and amino acid sequence at the fusion protein cleavage site utilized by OIE to assess the virulence of new isolates [1]. Twenty-four of the isolates were higly virulent or velogenic and four were of moderate virulence or mesogenic, but all were of a virulence that makes their occurrence a reportable disease event and were the apparent cause of the ND outbreaks that were the source of the isolates. One difference among the isolates was that they can be categorized into two groups based on the fusion protein cleavage site motif: group 1 has the motif ^{112}

RRQRR^{116} and includes strains isolated between 1998 and 2001 and group 2 contains the motif ^{112} RRQKR^{116} and includes strains isolated between 2003 and 2005.

Phylogenetic analysis (Fig. 2) grouped all the isolates in genotype VII, the most prevalent NDV genotype in Asia since the 1980s [37, 38]. Yang et al. [39] reported that K^{101} and V^{121} are the unique features of the fusion protein for genotype VII based on a study that included primarily genotype VII isolates from Taiwan. A further study of genotype VII NDV isolates from Japan, China, Taiwan, and South African by Abolnik et al. [18] found that K^{101} and V^{121} were typical of the VIId subtype, whereas R^{101} was more typical of the isolates that group as subtype VIIb. The latter findings support the results of the present study which separated the Kazakhstan and Kyrgyzstan isolates into the VIIb and VIId subgenotypes.

Fourteen of the 28 NDV isolates of this study were found to be typical of genotype VIIb strains. This result extends the evidence reported by Bogoyavlenskiy et al. [16] by identifying additional outbreaks of ND occurring in Kazakhstan during 1998 caused by genotype VIIb viruses and that viruses of that genotype continued to cause outbreaks there during 2000 and 2001. It was notable that the primary change between the 1998 and 2000–2001 isolates was a change of F^{19} to V^{19}. The isolates of genotypes VIIb from GenBank appear to form four further distinguishable clusters [2], isolates mainly originating from Europe, from South Africa and Mozambique, Portuguese isolates and a Bulgarian isolate, and originating from Asia and America, respectively. All isolates from Kazakhstan during 1998–2001 were classified as belonging to the European cluster of VIIb genotypes and were most like FIN-1-96, an isolate from a goosander in Finland during 1996 [14]. However, the VIIb Kazakhstan isolates can be categorized into two groups: group 1 including all 1998 strains that have the residue F^{19} and group 2, the 2000–2001 isolates with V at position 19.

The 14 most recent isolates from Kazakhstan and Kyrgyzstan recovered during 2003–2005 were classified as genotype VIId, a genotype recovered at a high frequency particularly in Asia during recent years [37, 38]. The genotype VIId isolates from Kazakhstan and Kyrgyzstan were more closely related to Chinese isolates (China-B1F4 and China-GIF3) (Figs. 1, 2) rather than Japanese and Taiwan isolates. Amino acid fusion protein sequence A^{11}, L^{28}, V^{52}, R^{78}, K^{101}, and K^{115} differentiated the VIId isolates from the VIIb isolates in the study.

Although avian flocks in Kazakhstan and Kyrgyzstan are regularly inoculated with attenuated vaccines, ND outbreaks still occur frequently [36]. A comparison of isolates that caused the outbreaks in the 1980s with the current isolates has not been done; therefore, it is unknown whether outbreaks from 1998 are from virus that became endemic in the poultry population or whether new introductions have occurred. The strong similarity of the 1998, 2000, and 2001 isolates with an isolate from waterfowl (FIN-1-96) and the similarity of the 2003–2005 isolates to chicken isolates from China (China-BIF4 and China-GIF3) are consistent with the possibility that the recent introductions were from migratory birds and legal or illegal trade, respectively. It has recently been demonstrated experimentally that the best protection against ND is with vaccines that are of a homologous genotype to the virulent challenge that vaccinated birds are likely to experience. Most notable is that a bird shed fewer viruses after challenge when the vaccine is homologous to the challenge and therefore the potential of spread from infected vaccinate is reduced [40]. Since the ND vaccines most widely used world wide are low-virulence viruses of genotype II [3], birds that become infected with genotype VII viruses, like the isolates in this study, are likely to shed more virus which would enhance the potential of spread to other susceptible birds. While it is theoretically possible that birds that are minimally protected by vaccination provide the potential for virus mutations, it seems less likely that is the source of the virulent viruses evaluated in this study. The importance of developing vaccines that express antigens homologous with the circulating virulent strains to provide optimal protection is widely recognized. To that end, a recent report describes the improved efficacy of a genotype VII vaccine created by reverse genetics when compared to the standard genotype II ND vaccines [41].

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